Review Article

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Toxicity of ethylmercury (and Thimerosal): a comparison with methylmercury

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ABSTRACT: Ethylmercury (etHg) is derived from the metabolism of thimerosal (o-carboxyphenyl-thio-ethyl-sodium salt), which is the most widely used form of organic mercury. Because of its application as a vaccine preservative, almost every human and animal (domestic and farmed) that has been immunized with thimerosal-containing vaccines has been exposed to etHg. Although methylmercury (meHg) is considered a hazardous substance that is to be avoided even at small levels when consumed in foods such as seafood and rice (in Asia), the World Health Organization considers small doses of thimerosal safe regardless of multiple/repetitive exposures to vaccines that are predominantly taken during pregnancy or infancy. We have reviewed *in vitro* and *in vivo* studies that compare the toxicological parameters among etHg and other forms of mercury (predominantly meHg) to assess their relative toxicities and potential to cause cumulative insults. *In vitro* studies comparing etHg with meHg demonstrate equivalent measured outcomes for cardiovascular, neural and immune cells. However, under *in vivo* conditions, evidence indicates a distinct toxicokinetic profile between meHg and etHg, favoring a shorter blood half-life, attendant compartment distribution and the elimination of etHg compared with meHg. EtHg's toxicity profile is different from that of meHg, leading to different exposure and toxicity risks. Therefore, in real-life scenarios, a simultaneous exposure to both etHg and meHg might result in enhanced neurotoxic effects in developing mammals. However, our knowledge on this subject is still incomplete, and studies are required to address the predictability of the additive or synergic toxicological effects of etHg and meHg (or other neurotoxicants). Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: Ethylmercury; methylmercury; Thimerosal; neurodevelopment; fish

Introduction

The chemical forms of mercury and its main toxic features have been amply reviewed, and an authoritative discussion can be found elsewhere (Clarkson and Magos, 2006; Magos and Clarkson, 2006). Ethylmercury (etHg) is an organic mercury compound derived from the metabolism of mercury [(o-carboxyphenyl) thio]ethyl sodium salt (thimerosal)) in the human body. Because of the widespread exposure to repetitive low doses of etHg through thimerosal-containing vaccines (TCVs) at very early ages of human development (Marques et al., 2007), including pregnancy and postnatal vaccination schedules, there is special interest in its comparative toxicology. Thimerosal remains the most widely used chemical form of organic mercury. Because of its use as a preservative in vaccines, almost every human (and animal) that has been immunized using TCV has been exposed to etHg. In contrast to methylmercury (meHg), which is an organic mercury source predominantly present in aquatic environments (seafood) and responsible for the poisoning of fish-eating populations (Clarkson and Strain, 2003), research on the specific toxicity of low doses of etHg relevant to vaccines has only recently been performed (Ball *et al*., 2001; Barile *et al*., 2012; Dórea, 2010, 2011). Accordingly, the role of multiple doses of TCV in current immunization schedules is a public health issue. From Table 1, it is evident that exposure to mercury in the form of etHg is lower than that from meHg in fish-eating populations. However, we should emphasize that thimerosal is injected intramuscullary, and consequently, its absorption is approximately 100% at a very early age (perinatally and during infancy) and in several doses (Dórea, 2007). The absorption of Hg (as meHg) after fish intake has been ideally estimated to be approximately 90%. Furthermore, fish meat typically contains nutrients that can counteract meHg toxicity, including selenium, which can form inert complexes with Hg (Clarkson and Strain, 2003; Davidson et al., 2011; Korbas *et al*., 2010; Pinheiro *et al*., 2009) and 3-n polyunsaturated fatty acids (Kris-Etherton *et al*., 2002).

Alkylmercurials such as meHg and etHg are toxic across a wide range of species (Hempel *et al*., 1995). These toxic features have been critical for the development of proprietary biocide formulas that are extensively used in agriculture and medicine. However, as a result of their environmental impact on wild life and accidental human disasters, the use of organomercurials as fungicides (90% meHg; 10% etHg) was banned in the early 1970s (Westermark *et al*., 1975). Mercury's use in medicine has a long history. In modern medicine, its use as an antimicrobial agent (topical antiseptic solutions, ointments, a preservative in vaccines, and ophthalmic products and cosmetics) has been significantly reduced or banned in many countries. However, mercury is currently employed as thimerosal in multi-dose vials of some vaccines. Except for the wealthier countries of North

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Table 1. Approximate mercury (Hg) exposure after typical schedules of vaccination in new born infants and hypothetical maternal exposure (during pregnancy and lactation) to Hg after consumption of different types of fish

Thimerosal			
Age of exposure		Approximate dose of Hg	
2 months		0.050 mg[a]	
6 months		0.110 mg[a]	
18 month		0.240 mg[b]	
14 weeks		0.190 mg[c]	
Methylmercury			
		fish	
Frequency	Sardine[d,e]	Salmon[d,e]	Shark[e]
Sporadic fish intake (less than 1 kg per year)	0.01 mg	0.020 mg	0.5 mg
Moderate fish intake (about 12 kg per year)	0.12 mg	0.24 mg	6 mg
Frequent fish intake (about 48 kg per year)	0.48 mg	0.96 mg	24 mg

[a]Pichichero *et al*. (2002)
[b]Redwood *et al*. (2001)
[c]Clements *et al*. (2000)
[d]Roos *et al*. (2012)
[e]Smith and Guentzel (2010)

America and the EU, TCVs (multi-dose vials) are prescribed to pregnant mothers and infants worldwide.

Although there are many studies on organic mercury toxicity with meHg, which reaches humans mainly through fish and seafood consumption, there are relatively fewer studies involving etHg. The main objective of this review was to integrate findings of *in vitro*, *in vivo* and human observational studies that have compared etHg and meHg to assess the cellular and systemic metabolism and the relative toxic potential of cumulative insults.

Occurrence, Exposure and Models

Although the photoalkylation of Hg(II) in nature can produce both meHg and etHg (Yin *et al*., 2012), the occurrence of natural etHg is only of academic interest. Among the two possible naturally occurring organic mercury species, meHg is the only one that is abundant and detectable in the water compartment and in living creatures, including fish and seafood for human consumption (Park *et al*., 2011; Zhao *et al*., 2012). Methylation is a common reaction in biology and, in several instances, occurs as an adaptive way to decrease the toxicity of reactive electrophilic compounds (Alix, 1982; Levander, 1977; Lohr *et al*., 1998; Pan-Hou and Imura, 1982). In contrast, ethylation is rare in biology. In normal cell physiology, methylation is largely dependent on methyl group transfer from the natural amino acid methionine (via the universal methyl group donor, S-adenosyl-methionine or SAM) to different methyl-accepting biomolecules. The analogous ethylation reaction using ethionine (a non-natural amino acid analog to methionine) as an ethyl group donor has serious toxicological consequences and highlights the fact that ethylation is not important in cell physiology (Alix, 1982).

Although not well studied from a pure chemical point of view (i.e. no biological catalyst is concerned), the stability of meHg in mammalian cells is greater than that of etHG (for an unequivocal demonstration of this event, see Burbacher *et al*., 2005 and Fig. 1). This fact will become apparent later in this review, and the higher stability of meHg over etHg significantly affects the different fates of the Hg after exposure to these alkylmercurials.

Conversely, the occurrence of meHg in vaccines (the potential conversion of etHg into meHg) is thermodynamically unlikely/impossible (Gibicar *et al*., 2007). Therefore, understanding the importance of these compounds in the comparative toxicology of organic mercurials requires knowledge of their differences in terms of occurrence and exposure. While meHg exposure occurs via seafood and, to a lesser extent, in rice in parts of Asia (Li *et al*., 2010), where it is expected to be bound to thiol (-SH) and selenol (-SeH) protein matrices, the etHg in vaccines occurs as part of thimerosal and is used as a preservative [in this molecule, etHg is bound to a thiol (sulfur) atom in the thiosalicylic acid (Fig. 2A)].

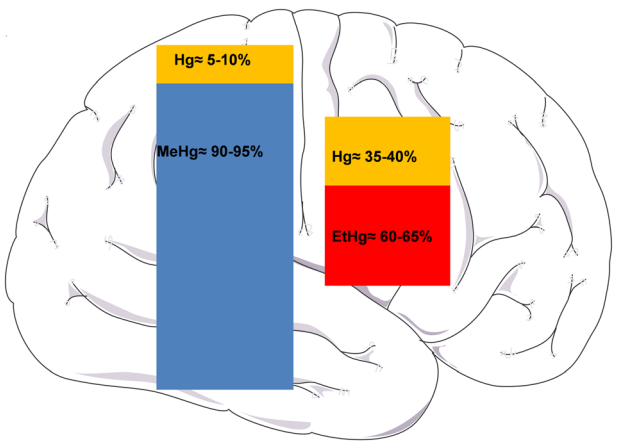

Figure 1. Schematic representation of the conversion of methylmercury (meHg, indicated in blue) and ethylmercury (etHg, indicated in red) to Hg (inorganic Hg, indicated in orange) after the administration of a similar dose of meHg (orally) or etHg (intramuscularly) to monkeys. The percentages are qualitative approximations of the data presented in Burbacher *et al*. (2005) for mercury levels in the brain of *Macaca fascicularis*, which were determined within 1 week of oral (meHg, left rectangle) or intramuscular (etHg, right rectangle) administration. The size of a rectangle represents a rough approximation of the total Hg retained in the brain of the monkey within 1 week of exposure.

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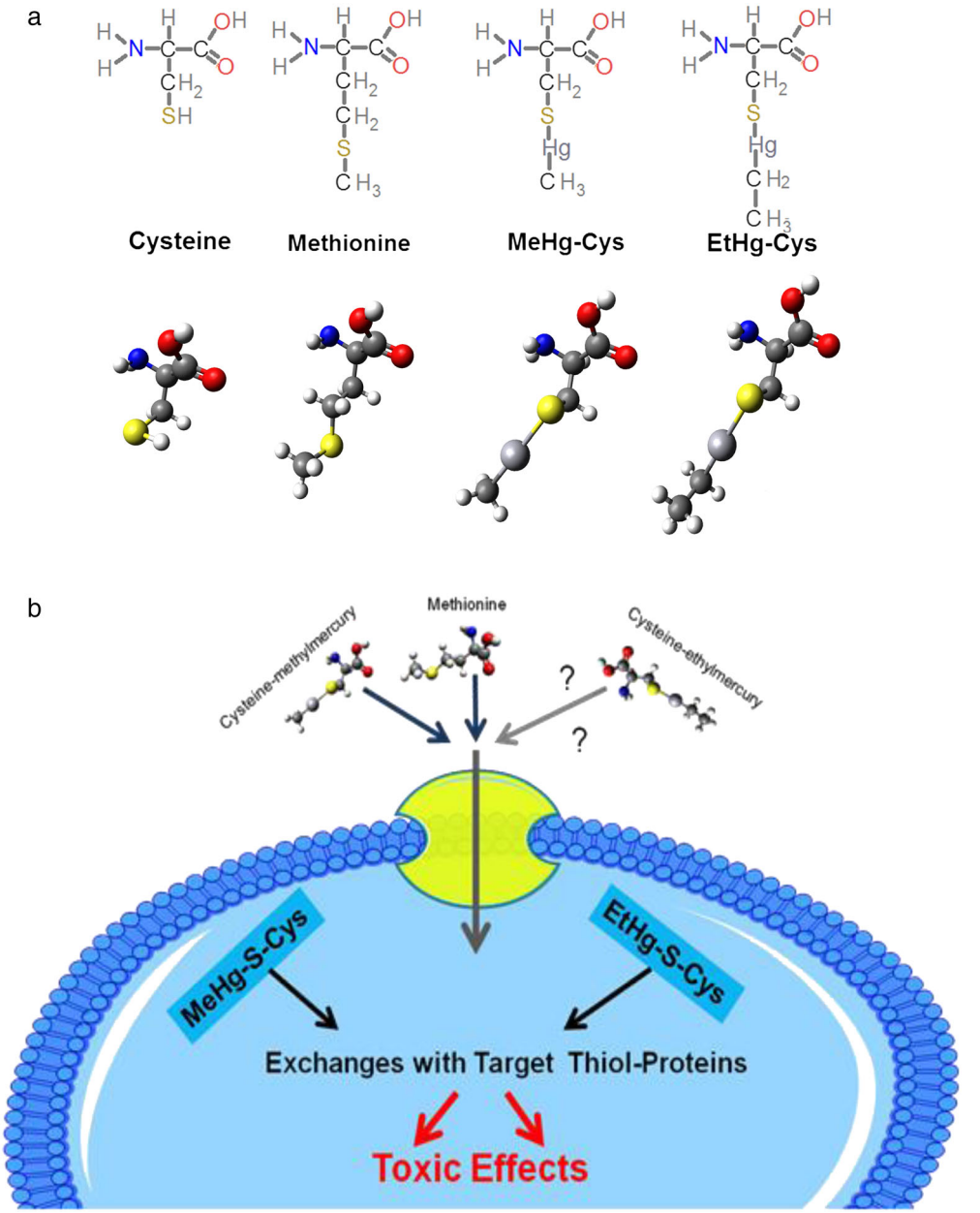

Figure 2. (A) Molecular mimicry of methylmercury (meHg) and ethylmercury (etHg) transport. MeHg can move inside different types of mammalian cells using a molecular mimicry mechanism. After forming a stable bond with cysteine, the MeHgS-Cys complex can be transported by the transporter protein involved in the uptake of methionine (see Ballatori, 2002; Bridges and Zalups, 2005; Roos *et al.* 2011; Yin *et al.* 2008). The exact region of the complex that is recognized by the transporter is unclear (Hoffmeyer *et al.*, 2006); however, experimental studies supporting this mimicry mechanism of MeHg-S-Cys are well documented in the literature (see Aschner, 1989; Aschner and Clarkson, 1988; Aschner *et al.*, 1990; Ballatori, 2002; Bridges and Zalups, 2005). EtHg may also be transported by a similar mimicry mechanism (unpublished data); however, experimental evidence is still lacking in the literature. (B) Schematic representation of methionine, the methylmercury–cysteine complex (MeHg-S-syC) and the ethylmercury–cysteine complex (etHg-S-syC) via the plasma membrane transporter protein (L-neutral amino acid transporter protein or LAT; Yin *et al.*, 2008).

The toxicological importance of organic mercury substances is related to their respective chemical properties in tandem with their ability to permeate cells and body compartments and undergo biological transformations. Hempel *et al.* (1995) tested organomercurial compounds with a battery of bioassays to assess and compare general toxicity and, in particular, genotoxicity (resazurin reduction method, *Spirillum volutans* test, nematode toxicity assay using *Panagrellus redivivus*, Toxi-Chromotest and SOS Chromotest). A ranking of the toxicity of the organomercurials demonstrates that MeHg > EtHg > Hg. This sequence of toxicity has been observed in LD_{50} toxicity assays using rainbow trout and in cytotoxic tests using bluegill-sunfish epithelioid cells (Babich *et al.*, 1990).

It is recognized that the neurotoxic properties of mercurials depend on their capability to enter the central nervous system (CNS). In this regard, in addition to lipophilicity, the presence of specific transporters at the blood–brain barrier and events such as 'mimicry' also play important roles in mediating the transport of mercurials from blood to the CNS (Ballatori, 2002). This phenomenon has been well documented for meHg

(Aschner and Clarkson, 1988; Aschner *et al*., 1990; Ballatori, 2002; Bridges and Zalups, 2011; Roos *et al*., 2011, 2012), whereas little is known about the transport of etHg by mimicry transport processes (Fig. 2). When considering the strong affinity of both organic forms of Hg for thiol groups, after getting into a living cell, they are expected to be found almost completely bound to the –SH groups of biomolecules (see Fig. 2). In the case of etHg, which is typically injected intramuscularly, its redistribution through the body will depend on a complex exchange reaction between intramuscular target proteins, low-molecular-weight intracellular thiol molecules (e.g. cysteine and reduced glutathione), thiol proteins in the plasma membrane and high- (proteins) and low-molecular-weight thiol molecules in the interstitial fluid. The etHg bound to thiol groups (low- and high-molecular-weight forms) in the interstitial fluid will sequentially be exchanged or transported to capillary cells and then, by similar chemical and biochemical processes, to the plasma, where it will be bound predominantly to albumin (which is the most plentiful protein in the plasma). For clarity, the distribution of Hg in Fig. 2A is depicted as etHg moving from inside the skeletal muscle to plasma and then to its main target tissues. Hg is similarly internalized from fish protein (as Cys-$HgCH_3$) into gastrointestinal cells and is then directly transferred to plasma (Fig. 2B; for clarity, the steps involved in the transfer of meHg from cells to the interstitial fluid to capillaries were omitted).

The binding of mercury species to plasma proteins is at the core of its metabolism and biologic effects. Li *et al*. (2007) compared inorganic Hg with different organic mercury compounds (etHg, meHg and phenylmercury) that bind with human serum albumin (HSA). The stoichiometries for the interactions of Hg^{2+}, $meHg^{+}$, $etHg^{+}$ and phenylmercury $^{+}$ with HSA were found to be 6:1, 4:1, 4:1, and 3:1, respectively. In addition, there are also biochemical differences in mercury-binding proteins between animal species. Janzen *et al*. (2011) compared the hemoglobin of human and rat origin and demonstrated that rat hemoglobin possesses twice as many free thiol groups. These authors concluded that there are different binding behaviors for human and rat hemoglobin and, therefore, a limited comparability between humans and rats in this instance.

As first theorized by Janzen *et al*. (2011), the difference between human and rat hemoglobin is of particular importance for the study of mercurials in vertebrates. However, it must be emphasized that we know very little about the molecular fate of Hg in the body. After coming into contact with a biological system, electrophilic (cationic) forms of Hg (Hg^{2+}, $meHg^{+}$ and $etHg^{+}$) are expected to rapidly bind to thiol (sulfhydryl or –SH) groups. In the case of $meHg^{+}$ derived from fish, $meHg^{+}$ would be bound to the cysteine residues of the fish protein (for a review, see Farina *et al*., 2011a and Fig. 3). For etHg, its fate is expected to be different. After injecting thimerosal, the released etHg would be expected to bind to small (cysteine, glutathione) and large (proteins) endogenous thiol-containing molecules. However, we know very little about this process and less about how the bound etHg is distributed throughout the blood and body.

Additionally, specific human, rat, guinea pig and rabbit phagocytic cells (polymorphonuclear leukocytes, human monocytes and guinea pig eosinophils) display dealkylating differences. Under identical conditions, meHg degradation by these cells is always much weaker than etHg (Fang and Fallin, 1974; Suda *et al*., 1992). Although these two studies have contributed important concepts to this field, our current knowledge about the chemical and biochemical stabilities of these alkylmercurials is only basic. In fact, there are important gaps in the field of mercury chemical and biochemical toxicology that are critical to better understanding the subtle toxicity of these contemporary forms of Hg exposure.

In Vitro Studies

Different *in vitro* approaches have been used to compare the toxicities of organomercurials (predominantly meHg and etHg). These approaches are mainly based on the use of isolated cells (*ex vivo* studies) and on cultured cell or slice approaches.

Non-Neural Cells

Few studies have compared the toxicity of organomercurials in non-neural cells. Cellular viability and nucleic acid synthesis have been studied in HeLa cells, and etHgCl is slightly more toxic than meHgCl (Chao *et al*., 1984). Thimerosal and meHg are capable of increasing intracellular Zn^{2+} concentrations in rat thymocytes (Hashimoto *et al*., 2009). Increases in intracellular (NG-108-15 cells) or intrasynaptosomal Zn^{2+} concentrations that are mediated by meHg were first observed approximately 15 years ago by Denny and Atchison (1994, 1996). Of toxicological significance, Zn^{2+} is central to cell death processes such as apoptosis and necrosis. This same research group demonstrated that meHg (at concentrations of 100 nM or higher) significantly decreased the intensity of 5-chlormethylfluorescein fluorescence, an indicator of cellular thiol content and increased intracellular Zn^{2+} concentrations (Kawanai *et al*., 2009). The concentrations of thimerosal required to increase intracellular zinc in rat thymocytes are higher than those of meHg (Hashimoto *et al*., 2009; Kawanai *et al*., 2009). In YAC-1 lymphoma cells, both meHgCl and thimerosal produce higher levels of down-regulation in calmodulin receptor expression than $HgCl_2$; however, thimerosal's effect on B-Tub-MFI was more pronounced (Yole *et al*., 2007).

Mercury compounds (mercuric chloride, meHg chloride and thimerosal) are effective in leading to the dose-dependent activation of phospholipase D (PLD) in monolayers of bovine pulmonary artery endothelial cells (BPAECs) in culture (Hagele *et al*., 2007). As compared with vehicle-treated cells, thimerosal induces a 2-, 6- and 4-fold activation of PLD at concentrations of 10, 25 and 50 μM, respectively, in BPAECs after 1 h of cell treatment. Activation was inhibited by D-penicillamine (a sulfhydryl-containing agent), which was more effective on thimerosal- than on meHg chloride-induced PLD activation (Hagele *et al*., 2007). The higher effectiveness of D-penicillamide probably indicates that thimerosal can directly react with D-penicillamide's –SH (thiol) group and that the etHg moiety could be directly transferred from thiosalicylate to the thiol group of D-penicillamide, resulting in a net lower concentration of free $R\text{-}Hg^{+}$ in the medium. Furthermore, the exchange of etHg from thimerosal to D-penicillamide's thiol groups could mitigate the mercurial interaction with its potential biological targets (see Fig. 3A). This same group (Peltz *et al*., 2009) demonstrated that calcium and calmodulin regulate mercury-induced PLD activation in BPAECs and that organic mercury (meHg > thimerosal) significantly activates PLD in BPAECs. In mouse AECs, similar observations have been made by Secor *et al*. (2011), who observed a dose- and time-dependent activation of PLD by meHg, etHg and Hg^{2+}.

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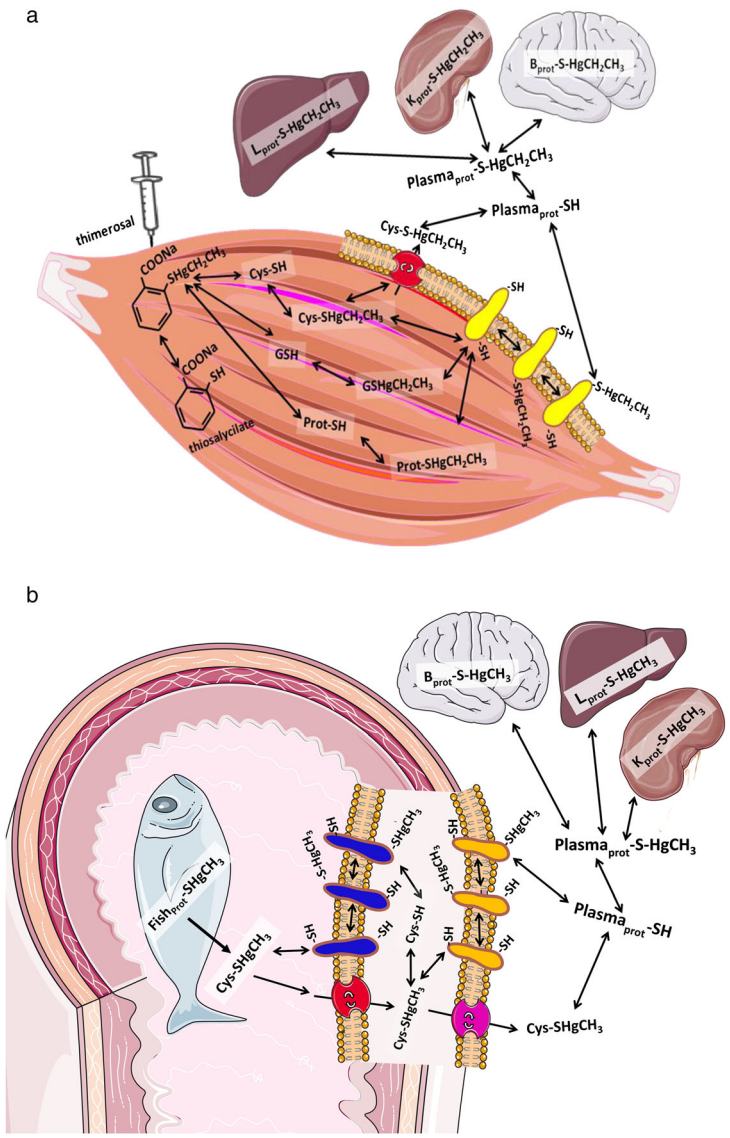

Figure 3. (A) Schematic representation of intramuscular ethylmercury administration [$CH_3CH_2Hg(II)$] as thimerosal. After thimerosal injection, etHg is released (owing to the disruption of the mercury-sulfur linkage in the thimerosal molecule) and interacts with thiol-containing proteins (Prot-SH) and low-molecular-weight thiols [mainly cysteine (Cys-SH) and reduced glutathione (GSH)]. Thimerosal also releases its non-Hg moiety (thiosalicylate), which presents no significant toxic effects. The etHg–cysteine ($Cys\text{-}SHgCH_2CH_3$) complex can be exported from muscle cells either by plasma membrane transport proteins (indicated by the unidirectional arrow through the red plasma membrane protein) via mimicry processes (see Fig. 2 for more details about molecular mimicry) or via exchange reactions with the plasma membrane thiol-containing proteins (indicated by bidirectional arrows with the yellow plasma membrane proteins). The etHg complexed with GSH ($GSHgCH_2CH_3$) can be exported via exchange reactions (indicated by bidirectional arrows). The exported etHg-SR complexes can then exchange with generic plasma thiol proteins (represented by Plasmaprot-SH) or with low-molecular-weight thiols (e.g. GSH or cysteine). For clarity, the transference steps (exchange or uptake via the mimicry process) between the low-molecular-weight thiol compounds (Cys-S-Hg CH_2CH_3 or GS $HgCH_2CH_3$) and the plasma and tissue thiol-containing proteins (B_{prot}-SH, K_{prot}-SH and L_{prot}-SH) have been omitted. The exchange steps involving the thiols from the interstitial fluid with those of the cell's plasma membrane or low-molecular-weight thiol–etHg complexes have also been omitted (note that equivalent steps occur between the muscle cells and their surroundings and between the plasma proteins, endothelial cells, erythrocytes and other blood, plasma membrane, and thiol-containing proteins). These low- or high-molecular-weight complexes are found in the interstitial fluid and can then be exchanged with generic mercury target kidney (K_{prot}-S-$HgCH_3$), liver (L_{prot}-S-$HgCH_3$) and brain (B_{prot}-S-$HgCH_3$) proteins. The transformation of CH_3CH_2-Hg^+ to Hg^{2+} and the subsequent binding of Hg^{2+} to low- and high-molecular-weight thiol molecules has been omitted. The metabolism of etHg to cationic Hg is expected to drastically alter the fate of Hg in the body (for a comprehensive review regarding inorganic Hg transport, see Bridges and Zalups, 2011). (B) Schematic representation of Hg (as MeHg) absorption in the gastrointestinal system. MeHg bound to fish thiol-containing proteins is released after digestion as a complex of MeHg–cysteine ($Cys\text{-}SHgCH_3$). The complex can then be transported to the interior of intestinal cells by either an exchange reaction (indicated by bidirectional arrows; see Farina *et al*. 2011a; Rabenstein *et al*., 1982, 1983, 1986) or via integral membrane transport proteins (indicated by unidirectional arrows) by mimicry processes (see Fig. 2 and Aschner *et al*., 1990; Ballatori, 2002; Bridges and Zalups, 2005; Roos *et al*., 2011; Yin *et al*., 2008). The meHg–Cys complex can be exchanged either with low-molecular-weight thiols (e.g., cysteine or GSH) or with different thiol-containing proteins [represented by the dark blue, orange, generic plasma protein ($Plasma_{prot}$-SH) or generic mercury target kidney (K_{prot}-S-$HgCH_3$), liver (L_{prot}-S-$HgCH_3$) and brain (B_{prot}-S-$HgCH_3$) proteins]. As indicated for etHg, several steps involved in the exchange and transport between the cells and body fluids have been omitted (e.g. lymphatic system, interstitial fluid, endothelial and blood cells).

During an *in vitro* study using monocytes from healthy donors, InSug *et al*. (1997) observed that different organic mercurials induced apoptosis, which was related to changes in lipid organization within the plasma membrane. However, this event was significantly dependent on the concentration of mercury in the medium, regardless of the chemical form of the metal (methyl, ethyl or phenyl mercury), and no distinguishable difference between the chemical form of the mercury was observed.

At similar concentrations, Migdal *et al.* (2010b) demonstrated that etHg induces a more pronounced human (monocyte-derived) dendritic cell activation response than meHg; however, thiosalicylic acid (the non-mercury moiety of thimerosal) had no effect. Interleukin (IL)-8 and tumor necrosis factor-alpha (TNF-α) were the main cytokines secreted after treatment. These authors also demonstrated that thimerosal and ethylmercury induce oxidative stress (Migdal *et al*., 2010a).

In harbor seals from the North Sea, Kakuschke *et al*. (2009) demonstrated that both measures of the cellular immune system (lymphocyte proliferation and cytokine expression) were influenced by Hg *in vitro*. These differences were dependent on the duration of exposure, animal age and chemical form of Hg. Cells obtained from young seals displayed a higher influence of both meHg and etHg. The degree of Hg-induced inhibition of lymphocyte proliferation by meHg was more immunotoxic than that by etHg. Yaqob *et al*. (2006) estimated the clinical relevance of the lymphocyte proliferation test as a potential diagnostic tool for detecting metal-induced inflammation in patients with symptoms of chronic fatigue syndrome. These authors demonstrated a reduced ability of lymphocytes to respond to metals *in vitro* after replacing metal dental implantations, which appeared to be more accentuated for thimerosal than for meHg (Yaqob *et al*., 2006).

Gardner *et al*. (2010) compared inorganic and organic species (meHg and etHg) of Hg with regards to their effects on human peripheral blood mononuclear cells (PBMCs) *in vitro* and at sub-cytotoxic concentrations and reported that both chemical forms of Hg, organic and inorganic, can affect the human immune system. However, the forms exerted different effects. Whereas inorganic Hg and meHg increased pro-inflammatory cytokine release in LPS-stimulated PBMCs, etHg decreased IFN-6 release and pro-inflammatory cytokine release. These results are somewhat difficult to explain and might indicate that a small difference in chemical reactivity can elicit a disparate response after LPS stimulation or that the interaction with thiols is not important in the evaluated parameters.

It appears that the biological effects of different mercury compounds on immune cells are not uniform. In addition, it is noteworthy that some of the above-mentioned studies compared the *in vitro* toxicities of meHg with thimerosal but not with etHg. Although it is well established that thimerosal is rapidly converted under *in vivo* conditions into etHg, whose 'molecular fate' is, to some extent, expected to be similar to that of meHg, the occurrence of a dissimilar rate of etHg formation after thimerosal exposure under *in vitro* conditions cannot be ruled out. As discussed above, our knowledge of the specific molecular fate of meHg, etHg and Hg^{2+} is still limited even when simple *in vitro* systems such as cultured lymphocytes are considered. Of interest, the toxicity of thimerosal toward kidney cells can be attributed predominantly to its Hg moiety (Park *et al*., 2007). In fact, the faster the decomposition rate of etHg, compared with meHg, to Hg(II) can determine the preferential targeting of kidneys by thimerosal.

Neural Cells

EtHg and meHg are potent cell disruptors, targeting a vast array of cellular regulatory pathways capable of inflicting various degrees of cell damage or disruption by multiple mechanisms. Although a complete understanding of the mechanisms mediating mercury-induced neurotoxicity is not yet available, this question has been better explored for meHg than etHg. Among the critical phenomena that mediate meHg neurotoxicity, the most important are the depletion of intracellular antioxidants, the inhibition of critical enzymes and the modulation of the activity of transporter and neurotransmitter or neuromodulator receptor activity (for a review, see Farina *et al*., 2011a,2011b). With respect to etHg, scarce mechanistic data are available; however, thimerosal and etHg have been reported to cause oxidative stress in monocytes (Migdal *et al*., 2010a; see above for more details).

Comparative experimental studies on the neurotoxic properties of etHg and meHg have provided no uniform data, which may be mainly related to the different animal species used and different exposure schedules (duration of the exposure, dosages, and period of exposure). Zimmer *et al.* (2012) demonstrated the potency of thimerosal in reducing cell migration in a human neural crest cell model and found that the toxicity of thimerosal was higher or at least comparable to meHg.

Hewett and Atchison (1992) studied the potential blockage of terminal nerve calcium channels using various mercurials (meHg, etHg, Hg^{2+}, dimethyl mercury, p-chloromercuribenzoate and p-chloromercuriphenylsulfonate). These authors found that mercurials with dissimilar charges and lipophilicities differentially affected synaptosomal Ca^{2+} influx. However, Ca^{2+} influx was blocked by both meHg and etHg, and these monovalent species also blocked Ca^{2+} influx into synaptosomes in a voltage-dependent manner. In that study, high concentrations of mercurials were required to inhibit Ca^{2+} influx into synaptosomes, and consequently, their blockage is likely not important in mediating meHg and EtHg toxicity (Hewett and Atchison 1992). Of note, the blockage of Ca^{2+} channels by different antagonists can protect rats (Sakamoto *et al*., 1996) and neurons from meHg neurotoxicity (Ramanathan and Atchison, 2011), suggesting the involvement of this ion in mercurial neurotoxicity. However, *in vitro* meHg inhibited cerebellar granule cell migration by decreasing the frequency of cytosolic Ca^{2+} spikes (Fahrion *et al*., 2012). In contrast, thimerosal enhanced cytosolic Ca^{2+} oscillations in human cardiac fibroblasts (Chen *et al*., 2010). Taken together, these results indicate that the interactions of mercurials with Ca^{2+}-handling proteins are complex and currently only partially deciphered (Roos *et al*., 2012).

Rush *et al*. (2009) tested the effects of metal chelators (EDTA, D-penicillamine, 2,3 dimercaptopropane-1-sulfonate and dimercaptosuccinic acid) on mercury (inorganic mercury, meHg and thimerosal) toxicity in cultured cortical cells. These authors reported that chelator protection was specifically provided only against inorganic mercury and that the chelators did not work against organic mercury (meHg and thimerosal). In that study, the percent of cell death was indistinguishable between meHg and etHg (Rush *et al*., 2009).

In line with that study, unpublished results from our group have also shown that meHg and etHg are similarly effective in inducing cell death and glutathione depletion in rat glioma cell cultures. Although some previous studies have demonstrated that both meHg and etHg present similar toxicities in *in vitro* experimental models of neurotoxicity, these results cannot be

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extrapolated to *in vivo* conditions. Indeed, the toxic potential of meHg under *in vivo* conditions is higher than that of etHg, which might be a consequence of different metabolism pathways, excretion rates and, consequently, half-lives (Burbacher *et al*., 2005).

In Vivo Studies

While *in vitro* studies provide better answers regarding mechanisms of action, *in vivo* experiments reveal more of the toxicokinetics (uptake by organs, transport, biotransformation and body elimination) and the toxicodynamics (specific and systemic effects, and functional outcomes) of substances of interest.

Toxicokinetics

Understanding the uptake and elimination rates of organic forms of Hg and their attendant transfer efficiencies into tissues is crucial for assessing the biological effects of these substances. These studies are essential for modeling the concurrent impacts of current organic forms of Hg (etHg and meHg) exposure on infant development.

Animals

Studies that compare organo-mercurials have reflected interest in different routes/modes of exposure and doses. Early studies utilized oral routes and large doses, whereas more recent studies have used smaller doses and exposures that have included lower doses via intramuscular injections. Indeed, earlier studies using radiolabeled Hg demonstrated that long-term oral exposure to organic mercury in adult rats result in higher mercury levels in the kidneys after etHg exposure but lower levels in the brain, as compared with meHg-exposed animals (Ulfvarson, 1962). These pioneering results indicate that etHg is probably being transformed to Hg^{2+}, which has limited access to the brain and typically targets the kidneys. Brzeźnicka and Chmielnicka (1985) studied the metabolism of etHg and meHg given intragastrically to adult rats for 2 weeks and found that, regardless of the dose, inorganic Hg from the dealkylation of etHg and meHg was found in the kidneys. However, in brain, the concentration of inorganic mercury is always higher in animals treated with etHg when compared with equivalent doses of meHg (Brzeźnicka and Chmielnicka, 1985). Similar findings were also observed by Magos *et al*. (1985) in rats after gastric gavage of etHg and meHg; that is, inorganic Hg concentrations were higher in kidneys and the brain after five daily doses of etHg than after meHg treatment. As observed above, these results indicate that etHg is more easily transformed to Hg^{2+} and, consequently, targets kidneys more efficiently than meHg.

Although the transformation of etHg to Hg(II) can explain, in part, renal targeting after etHg injection, the distribution and interaction of etHg and Hg(II) within renal cells are not identical. Blanusa *et al*. (2012) observed that Hg retention in the blood and brain was higher after subcutaneous exposures of suckling rats to etHg than to Hg^{2+}. Furthermore, the urinary excretion of Hg after Hg^{2+} was higher than that observed after etHg intoxication.

Rodrigues *et al*. (2010) compared the metabolism of etHg (thimerosal exposure) and meHg in adult rats and observed that Hg remains in the blood longer in animals treated with meHg. Most of the Hg in the etHg-treated animals was inorganic, and there were also differences in the uptake and metabolism of the Hg in the brain tissue. In etHg-treated animals, most of the brain mercury (63%) was inorganic, and only 13.5% was etHg. These results are in contrast with the meHg-treated animals, where 91.2% of the mercury is meHg. When comparing the doses of Hg given, there was more inorganic Hg in the organs (brain, liver, heart and kidneys) of the thimerosal-treated animals.

Zareba *et al*. (2007) compared the tissue concentrations and the disposition and metabolism of thimerosal with that of meHg in neonatal mice (10 days postnatal) given single intramuscular injections of both substances and reported different patterns of tissue distribution and different rates of mercury decomposition for the studied organic compounds. The organic mercury levels in the brain and kidneys were predominantly lower in the mice treated with thimerosal than in the meHg-treated group. The opposite result was observed with regards to inorganic Hg concentrations; that is, in the young mice treated with thimerosal, the inorganic mercury concentrations in the brain were predominantly higher (only significant on day 2) than in the meHg-treated animals.

Harry *et al*. (2004) studied organic Hg (meHg and etHg/thimerosal) metabolism in neonatal mice (postnatal day 16) that were treated with meHg (oral gavage and intramuscular injection) and etHg (intramuscular injection) and also compared immature and adult (60-day-old mice) animals that were exposed to a 10-fold higher dose of Hg. These authors demonstrated that the organ (blood, brain and kidneys) distribution after an intramuscular dose was 30% of that observed for an oral dose of meHg. Significant differences also exist between immature and adult animals for the brain distributions of both etHg and meHg. Twenty-four hours after acute dosing, only a mean of 0.06% and 0.09% (respectively for thimerosal and etHg) was found in adult mice compared with 0.22% and 0.4% (respectively for thimerosal and etHg) in immature animals (Harry *et al*., 2004). The distribution and/or accumulation pattern was also influenced by the route of exposure.

Suzuki *et al*. (1963) demonstrated that etHg is proportionally higher in the liver and kidneys and lower in the brain when compared with meHg. However, once retained in the brain, etHg did not decrease as observed in other organs (Suzuki *et al*., 1963). It is interesting to note that the deposition of Hg in the liver and kidney can be explained by a faster transformation of etHg to Hg^{2+} when compared with meHg. In fact, the majority of the results discussed above clearly indicate that the 'biological' or 'biochemical' stability of the Hg-C bound in the etHg is lower than that of meHg (Fig. 1).

Burbacher *et al*. (2005) compared blood and brain Hg after exposure to meHg (oral gavage) and to vaccines containing thimerosal (intramuscular injection) in baby monkeys at birth and 1, 2, and 3 weeks postnatal. The blood half-lives after thimerosal were 2.1 and 8.6 days (corresponding to days 2 and 38 after injection), whereas that of meHg was 21.5 days. These types of exposures also produced total Hg concentrations in the brain that paralleled the blood half-lives, and the Hg concentrations were significantly higher in meHg-exposed baby monkeys than those exposed to thimerosal. Monkeys (*Macaca fascicularis*) exposed to thimerosal displayed slightly higher concentrations of cerebral etHg than Hg^{2+}. However, a substantially higher proportion of inorganic Hg was found in the brains of monkeys injected with etHg when compared with those treated with meHg (Fig. 1).

Korbas *et al.* (2008) reported that the accumulation patterns of both meHg and etHg did not vary in the lenses of zebrafish larvae, indicating that impairments of the visual process owing to mercury may also arise from direct effects on the ocular tissue. Tryphonas and Nielsen (1973) observed that pigs treated with elevated oral doses (0.19 to 0.76 mgHg kg^{-1} bw per day) of Hg for 60 and 90 days had severely affected tissues (neuronal necrosis, secondary gliosis, capillary endothelial proliferation and additional neuronal necrosis as a result of developing degenerative arteriopathy) and relatively high concentrations of mercury.

When comparing the cytoxicity of thimerosal with that of meHg in cerebellar neurons dissociated from 2-week-old rats, Ueha-Ishibashi *et al.* (2004) found similar effects (at concentrations ranging from 0.3 to 10 μM) when increasing the intracellular concentration of Ca^{2+}[Ca^{2+}(i)]. As briefly explained above, Ca^{2+} is one important factor involved in organic mercury toxicity; however, the exact role of Ca^{2+} in mercury toxicity is not yet known (Roos *et al.*, 2012). An increase in extracellular Ca^{2+} after meHg exposure is thought to be related to an increase in extracellular glutamate (for a review, see Aschner *et al.*, 2007). Recently, Duszczyk-Budhathoki *et al.* (2012) demonstrated that exposing suckling rats to thimerosal is associated with an increase in cerebral extracellular glutamate, indicating that excitotoxicity is common to the two types of mercurial exposure.

Humans

The toxicokinetics of meHg and etHg in humans have been studied in both adult and pediatric patients. In adults, blood etHg increases in all treated patients; however, the increases in total mercury concentrations are significantly larger (Barregard *et al.*, 2011). These authors also observed that blood concentrations of meHg (from fish consumption) were much higher than those of etHg when injected with TCVs (Table 1). The total blood Hg probably includes both chemical forms of organic Hg. In specific and limited cases of 'suspected mercury poisoning', the levels of both alkylmercurials (meHg and etHg) were found at similar concentrations in urine samples (Tsoi *et al.*, 2010).

Immediately after intramuscular vaccination with TCV, Pichichero *et al.* (2008) detected etHg and meHg in blood samples from neonates and infants. Additionally, speciation studies have demonstrated both meHg and etHg in the hair of vaccinated children (Dórea *et al.*, 2011b), and the levels of etHg were correlated with the time of vaccination of the infants, who had been immunized with TCV (Dórea *et al.*, 2011a).

Comparative Biological Effects Between etHg and meHg

Chmielnicka and Brzeinicka (1978) compared etHg and meHg and demonstrated an increase in metallothionein-like proteins that was highly correlated with the level of inorganic mercury. Physiological differences in the kinetics of alkyl-mercurials that render etHg elimination faster than meHg appear to affect the renal system by accumulating inorganic Hg^{2+} in renal tissues (Fang and Fallin, 1974; Suzuki *et al.*, 1963). In comparing the renal toxicities of the mercuric compounds given to adult rats by oral gavage, these authors demonstrated a focal damage to the proximal tubules of the kidney and that etHg produced a more diffuse pattern of damage throughout the kidney than meHg (Magos *et al.*, 1985). Magos *et al.* (1985) also demonstrated that, on an equimolar basis, etHg was less neurotoxic than meHg; however, their nephrotoxic symptoms and signals were similar. These authors reported no significant differences between the effects of meHg and etHg on the dorsal root ganglia or on coordination disorders (Magos *et al.*, 1985).

Most environmental Hg exposure is derived from maternal seafood consumption, and the attendant effects on the brain can only be detected after the child is old enough to be tested (Grandjean *et al.*, 1998). This also holds true for mothers exposed to TCV during a pregnancy and for the subsequent infant's immunization schedule with TCV (Dórea, 2007). Animal studies modeling an infant's exposure to both forms of mercury, meHg from maternal sources (*in utero* or breastfeeding) and etHg in TCV, are not directly comparable to human situations. Instead, our understanding of the differences and/or cumulative effects of organic mercury (meHg and etHg) is derived from hypotheses generated by population studies. Dórea *et al.* (2012) suggested an association between Hg exposure from different sources (meHg and etHg) and neurobehavioral tests at 6 months. Studies in Korea (Lee and Ha, 2012) and Poland (Jedrychowski, 2012) have shown that co-occurring etHg is an important factor to include in neurobehavioral tests during neurodevelopment.

Immunotoxicity

Havarinasab *et al.* (2004) studied thimerosal's effect on systemic autoimmunity in genetically susceptible mice after exposing them to inorganic mercury and concluded that the effect of thimerosal is similar to that of inorganic mercury and that, at equimolar doses, meHg displayed the weakest immunostimulating, autoimmunogen and IC-inducing effects (Havarinasab and Hultman, 2005). This same group demonstrated that the autoimmunogen effect of etHg in mice was as a result of Hg^{2+} formed from etHg in the body (Havarinasab *et al.*, 2007). These findings reinforced the high dealkylating rate of etHg compared with meHg, and also indicated that the quality of the Hg-induced autoimmune response is modified by its chemical form.

All three chemical forms of mercury (metallic mercury, inorganic mercury and organic mercury) can cause delayed contact allergies (Möller and Trofast, 1979). Audicana *et al.* (2002) studied the cross reactivities and tolerances of mercury derivatives (ammoniated mercury and merbromin) and thimerosal and reported cross reactivities among Hg derivatives and a good vaccine tolerance in patients allergic to thimerosal.

Santucci *et al.* (1998a, 1999) studied interactions among alkylmercury (etHg and meHg) compounds on the skin. These authors found that the positive reactions to thimerosal were as a result of etHgCl, the structural similarities between etHgCl and meHgCl were very similar, and the skin reaction was identical with either compound (Santucci *et al.*, 1998a). The majority of the patients (19 out of 21) that presented positive patch-test reactions to both etHg and meHg were re-tested with meHgCl and solutions containing meHgCl mixed with cysteine, glutathione, $ZnSO_4$, $MgSO_4$, $MnSO_4$, $ZnCl_2$, $MgCl_2$ and $MnCl_2$ along with thiol fragments (of metallothionein I and metallothionein II-Zn), cysteine, glutathione and Zn(II) salts that were able to abolish the positive reactions. Indeed, Santucci *et al.* (1998b) speculated that subjects who react positively to alkylmercurials have a constitutively reduced capability to metabolize organomercury compounds.

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Does TCV-etHG Exposure Contribute to Cumulative Insults That Increase the Risk of meHG Toxicity in Developing Infants?

Environmental studies have demonstrated that maternal exposure to fish meHg can have consequences on the neurological outcomes of newborns (Suzuki *et al*., 2010) and children (Karagas *et al*., 2012; Llop *et al*., 2012). Collectively, existing studies indicate subtle neurological effects as a result of TCV-etHg (Dórea, 2010). Environmental (fish meHg) and iatrogenic (TCV-etHg) mercury can reach neural cells, where it can deplete glutathione and induce apoptosis along with other disrupting effects on cell metabolism. Thus, short alkylmercurials target the brain, and consequently, brain physiology can be disrupted even after absorbing a low injury dose of these mercurials (Dórea, 2011). Therefore, exposure to low doses of meHg and etHg increases the risk of combined (additive or synergistic) neurotoxic insults (Suzuki *et al*., 2010). Furthermore, considering the faster transformation of etHg, compared with meHg, to Hg(II) in the brain and the increased half-life of Hg(II), compared with etHg, in the brain, the simultaneous exposure of humans to meHg and etHg is expected to expose brain cells to three chemical forms of Hg. Although the targets of these mercurials can overlap, they do not completely coincide, reinforcing the theory that simultaneous exposure to meHg and etHg can result in both additive and synergistic effects to human organs.

The potential consequences of repeated low-level acute exposures to TCV-etHg *per se* or in combination with meHg on developing humans are still a matter of debate. The effects of different forms of Hg on cardiovascular, neural and immune tissues are comparable in *in vitro* models. As a fetus or during breastfeeding, the developing infant can be exposed to all chemical forms of Hg (organic and inorganic) via the placenta or breast milk. Additionally, most infants are exposed to TCV etHg in most countries of the world. In circumstances of chronic exposure to environmental meHg (and other neurotoxicants), additional acute (and repeated) doses of etHg during fetal and infantile neurodevelopment should be thoroughly evaluated.

Currently, it appears that the ability to detect the cumulative insults of co-occurring etHg and Me-Hg in higher functions during neurodevelopment is significantly driven by factors that influence neuroplascitiy (positively or negatively; Suzuki *et al*., 2010). Environmental health workers have been issuing advisories on fish meHg consumption while mounting studies of etHg neurotoxicity (Dórea, 2011) demonstrate that regulators in the EU and North American countries were correct for banning/discontinuing alkyl-mercurials in agricultural, hygienic and medical products.

Much of the important biological and toxicological effects of low doses of etHg (tissue culture and animal experiments) are relatively recent and still limited. As noted by Ishitobi *et al*. (2010), most neurotoxicity studies on mercury are based on research assessing only one form at a time, predominantly meHg, and indicate that co-exposures to meHg and other forms (Hg vapor) can increase neurotoxic risks at levels relevant to human exposure. Therefore, significantly more studies will be required to assess combined exposures (meHg and etHg), which also include other commonly co-occurring neurotoxicants in fish (such as polychlorinated biphenyls) and vaccines (such as aluminum).

The World Health Organization (WHO) recognizes the importance of mercury exposure and the vulnerability of children (WHO, 2010). Concerns and recommendations regarding exposure to meHg in fish and etHg in vaccines are generally addressed by different experts – environmental scientists and disease-preventing infectologists, respectively. While environmental scientists recognize that the mercury compounds poses a threat for the healthy development of the world's children (WHO, 2010), the WHO Global Advisory Committee on Vaccine Safety considers that the risks of the etHg in vaccines remain unproven and that there is no evidence of vaccine-etHg toxicity to infants and children exposed to TCV (WHO, 2010); thus concluding that there is no reason to change current immunization practices with such vaccines (WHO, 2010, 2012). EtHg-induced toxicity represents an issue of continued reviewing, and following the precautionary measure of North-American and European regulatory bodies, it seems reasonable that efforts be dedicated to world widely develop alternative procedures of immunization based on either single-dose vials or on the use of alternative (nontoxic) preservatives.

Concluding Remarks

- The differences in mercury metabolism in different organs largely result from the binding capacities of mercury's chemical forms and from the stability of carbon–mercury linkages in organic mercurials.
- The neurotoxicity of etHg is similar to meHg in most '*in vitro* systems', but differences in the kinetics between these two compounds display differences in tested outcomes. However, an immunotoxicity is more pronounced and more common for thimerosal etHg.
- The differences in the toxicities of these two contemporary and relevant forms of Hg can be explained by the faster degradation (spontaneous or enzymatic) of etHg when compared with meHg and Hg(II). Because the targets of these mercurials do not completely overlap, a simultaneous exposure to meHg and etHg can have unpredictable additive and synergistic effects on developing and mature humans.
- Age and type of exposure, route, and attendant differences in mercury chemistry make toxicological comparisons with etHg and meHg useful in understanding the complexity of mercury metabolism but not sufficient to establish safety recommendations.
- Existing animal models demonstrate that etHg is less neurotoxic than meHg, but we still require adequate models to demonstrate whether repeated relevant doses of etHg in combination with different meHg background exposures have consequences on fetuses and infants.
- EtHg and meHg are different compounds and lead to different exposure levels and different toxicity risks.
- Although few animal models have compared the toxicity of etHg to meHg (at high doses), a few human observational studies have indicated that when simultaneous low doses of etHg and meHg exposure occur during the perinatal period, there are weak associations with neurodevelopmental outcomes. Consequently, further detailed studies with low levels of simultaneous exposure to meHg and etHg are required to establish the hypothetical no-observed adverse effect level (NOAEL) in experimental models using different endpoints of toxicity (from biochemical to neurobehavioral determinations). Most importantly, large epidemiological studies are also required to ascertain whether simultaneous exposure to alkylmercurials can have more than additive long-lasting neurotoxicological effects on children.

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